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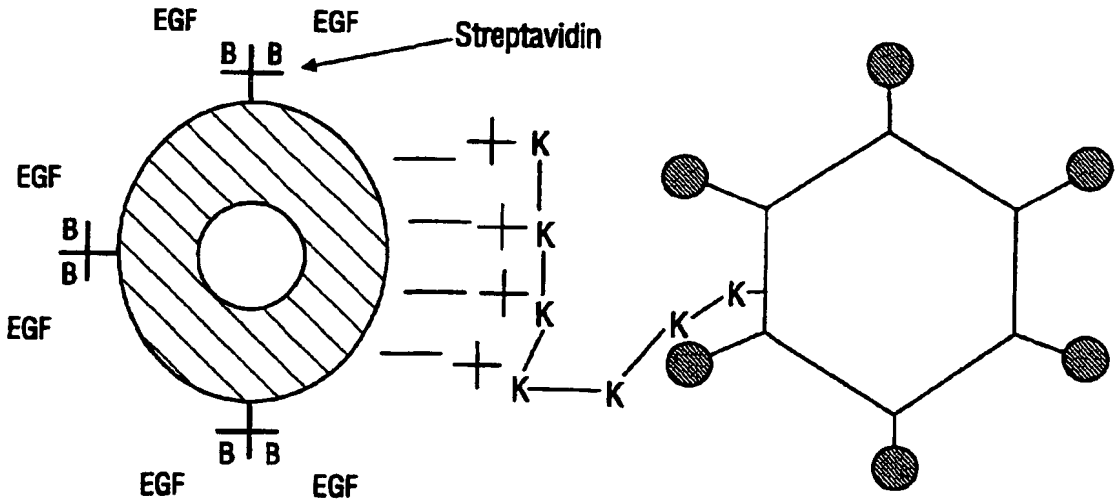

(57) Abstract

A receptor-mediated complex that selectively delivers nucleic acid into cells is disclosed. Epidermal growth factor (EGF)-receptor-binding peptide acts as a targeting ligand and is complexed with a component that binds nucleic acid. A reporter gene conjugated to EGF peptide/nucleic acid-binding agent was successfully transferred into a number of different cell lines that express high levels of receptor.

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DESCRIPTION

EGF-TARGETED NUCLEIC ACID DELIVERY

FIELD OF THE INVENTION

The present invention relates generally to the field of gene delivery. In particular, the invention relates to receptor-mediated gene targeting to lung cancer cells. In one example, the invention relates to epidermal growth factor (EGF)-mediated nucleic acid delivery and expression in lung cell lines.

DESCRIPTION OF THE RELATED ART

Lung cancer is one of the most frequently diagnosed malignant neoplasms throughout the world (Hammar, 1988). In the United States, lung cancer is now the leading cause of cancer death, killing more than 140,000 people annually. Age-adjusted mortality from lung cancer has recently surpassed that for breast cancer in women. Implementation of smoking-reduction programs has decreased the prevalence of smoking, but lung cancer mortality rates are expected to remain high well into the 21st century.

Although considerable research effort has been devoted to lung cancer therapy, the overall prognosis is poor, with present treatments such as surgery, radiotherapy and chemotherapeutics having a relatively limited effect (Hammar, 1988). Important considerations in effective therapy of lung cancer include a high selectivity in targeting cancer cells but not normal cells, *i.e.*, elevated levels of expression of a receptor on tumor cell surfaces, and efficient delivery of complete gene sequences into these target cells.

Recent advances in recombinant DNA and gene delivery technologies (Miller, 1992; Mulligan, 1993) suggest that gene therapy may be a promising alternative to standard radio- and chemotherapeutic regimens. The present vectors now available for gene delivery into lung cancer cells include recombinant retroviruses and adenoviruses. Each has been shown to work *in vivo*, but these vectors have limitations, such as the lack of cell-specific targeting, gene size limitations and safety concerns.

Another possible gene therapy approach, receptor-mediated gene targeting, takes advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993). Furthermore, in comparison with viral delivery systems (Morgan and Anderson, 1993), receptor-mediated gene delivery allows greater flexibility of DNA size and sequence because the DNA to be delivered does not need to be packaged into viral capsids. This helps avoid tedious clonal selection and virus-production processes. These characteristics make the system an attractive prospect for cancer therapy.

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994). *In vivo*, the distributions of ASOR and transferrin receptors are very different. Transferrin receptors are found in many different cell types, while

ASOR receptors are almost exclusively distributed on the sinusoidal domain of the hepatocytes (Wu and Wu, 1987).

Epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085). In this study, the DNA was coupled directly to an EGF receptor leaving the nucleic acid highly charged. This limits the size of nucleic acid that may be employed as it is difficult to link large molecules to ligands via a single covalent bond and its negative charge may reduce the efficiency of transfer due to the overall negative charge on cell surfaces. Furthermore, the integrity of the sequence may be altered or damaged during the process.

SUMMARY OF THE INVENTION

Therefore, it is an object of the present invention to provide improved compositions for use in gene therapy. It also is an object of the present invention to provide methods for the use of such compositions and, in particular, use in the treatment of cancer.

In satisfying these and other objectives, the present invention addresses the need for improved therapy for lung cancer and other diseases by providing compositions and methods for their use. The present invention encompasses a nucleic acid-binding agent/EGF complex that is capable of selectively delivering nucleic acid into cells via an EGF receptor uptake system. The present invention also provides compositions and methods to promote the uptake and expression of the nucleic acids in cells by use of an endosomal lysis agent.

In one embodiment, there is provided a complex comprising an epidermal growth factor (EGF) receptor-

binding peptide and a nucleic acid-binding agent. In preferred embodiments, the peptide is whole EGF, a fragment of EGF or a chemically synthesized portion of EGF.

In one embodiment, the nucleic acid-binding agent is a polycationic moiety. In a preferred embodiment, the nucleic acid-binding agent is polylysine. In further embodiments, the invention includes an endosomal lysis agent such as an infectious, replication-deficient adenovirus.

Complexes according to the present invention may further comprise a nucleic acid. The nucleic acid may be at least of a length selected from the group consisting of 10, 20, 50, 100, 500, 1000, 5000 and 10,000 base pairs.

In particular embodiments, the nucleic acid encodes a therapeutic gene. In a further embodiment, the therapeutic gene is operably linked to a promoter that is active in a cell expressing an EGF receptor. The therapeutic gene may be a tumor suppressor such as *p53* or *p16*. In other embodiments, the therapeutic gene may encode cystic fibrosis transmembrane conductance regulator (CFTR), blood clotting factor IX or other disease ameliorating proteins.

The nucleic acid may encode an antisense construct or a ribozyme. In a preferred embodiment, the antisense construct or ribozyme may target an oncogene transcript. In an even more preferred embodiment, the antisense construct or ribozyme targets a *ras* oncogene.

In further embodiments, the invention includes a pharmaceutical composition comprising a complex as described above and a pharmaceutically-acceptable

carrier, diluent or excipient. Another embodiment of the invention includes a kit comprising an EGF receptor-binding peptide and a nucleic acid-binding agent. In a preferred embodiment of the invention the ligand and nucleic acid-binding agent are complexed. The kit may further comprise a nucleic acid encoding a therapeutic gene. In a preferred embodiment, the ligand, nucleic acid-binding agent and nucleic acid are complexed.

Another embodiment of the invention includes a method of delivering a gene to a cell expressing EGF receptor. This method comprises providing a EGF-containing complex for the delivery of a gene to a cell and contacting said complex with said cell. In a further embodiment, a method of delivering a gene to a cell comprises contacting the cell with an endosomal lysis agent. In a preferred embodiment, the endosomal lysis agent is an infectious, replication-deficient adenovirus.

Another embodiment includes a method of treating a mammal with a lung tumor *in vivo* comprising providing a pharmaceutical composition as described above and administering the pharmaceutical composition to the mammal. Preferred embodiments of this method include the method where the cells of the lung tumor overexpress EGF.

Even further embodiments of the invention include a method of treating a mammal with a lung tumor *in vivo*, wherein said administering is selected from the group consisting of intravenous injection, subcutaneous injection, direct perfusion, inhalation, intratracheal injection and intra-peritoneal injection.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 Diagrammatic representation of the EGF/PLL/DNA complex in which EGF/Biotin (B) is linked to poly-L-lysine (PLL) by streptavidin. The interaction between PLL and the DNA is represented as a toroid structure, which occurs upon complete charge neutralization of the DNA.

FIG. 2 Diagrammatic representation of the EGF/PLL/DNA complex when it is coupled to the replication defective adenovirus that has been modified by the covalent attachment of PLL to the viral capsid. Coupling occurs between the positive charge of the lysine (K) groups and the negative charge of the DNA, resulting in the EGF/DNA/Adenovirus complex.

FIG. 3 Adenovirus enhancement of DNA delivery through the EGF/PLL/DNA complex, by using a non-modified adenovirus. The numbers of adenoviral particles per cell were 0, 30, 100, 300, 1000, 3000, and 10,000. Three micrograms of pCMV/β-Gal DNA in complex form with the EGF/PLL conjugate were incubated with the non-modified adenovirus. The percentage blue staining represents the percentage of cells staining positive for β-Gal expression. Cell lines; Open circle, SW620; Filled circle, H1299; Open triangle, H460a.

FIG. 4 Adenovirus enhancement of DNA delivery through the EGF/PLL/DNA complex, by direct attachment of

the complex to the E5 adenovirus. The numbers of adenoviral particles per cell were 0, 30, 100, 300, 1000, 3000, and 10,000. The amount of DNA incubated with the cells is equal to 0.000001 ng/adenoviral particle. The percentage blue staining represents the percentage of cells staining positive for β-Gal expression. Cell lines; Open circle, SW620; Filled circle, H1299; Open triangle, H460a.

FIG. 5 The quantitation of β-Gal expression in lung cancer cells after incubation with the EGF/PLL/DNA complex. The samples incubated with the cells are as follows: 3 μg EGF/PLL/DNA complex + adenovirus (Filled bar); EGF/PLL/DNA complex + E5 adenovirus (Open bar). In each sample 10^3 adenoviral particles/cell was used. The o-nitrophenol β-galactopyranoside analysis was done 24 h after the initial incubation with the cells. A unit is defined as nmol of o-nitrophenol formed per minute (Nielson *et al.*, 1983).

FIG. 6 Analysis of DNA delivery into various lung cancer cell lines by the EGF/PLL/DNA complex. The cell lines were incubated with the following: 3 μg EGF/PLL/DNA complex + adenovirus (Filled bar); EGF/PLL/DNA complex + E5 adenovirus (Open bar). In each sample 10^3 adenoviral particles/cell was used. Histologic staining was done 24 h after administration of the samples to the cells.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relies on the interaction between a specific receptor-ligand pair, namely, EGF and its cognate receptor. By attaching a nucleic acid to an EGF peptide, the peptide will direct the nucleic acid to cells expressing the receptor and initiate its uptake by

the cell. The attachment of the nucleic acid to EGF is not direct, however, but is facilitated by a nucleic acid-binding moiety that is, in turn, bound to EGF.

Throughout this application, the term "complex" is meant to include one or more molecules bound together by any physical or chemical means and encompasses the terms "conjugate" and "couple." The complex may be tightly or weakly bound together in a highly specific or totally non-specific way.

The term "ligand" is meant to include any molecule that binds to another molecule or macromolecule, the latter being a "receptor." The phrase "nucleic acid-binding agent" is intended to refer to any molecule or compound that binds a nucleic acid. A ligand and nucleic acid-binding agent may be complexed by any means including: covalent, ionic, hydrophobic, intercalative, van der Waals' or a combination of these forces and others.

EGF Peptides

According to the present invention, the targeting ligand is epidermal growth factor (EGF) or another EGF receptor-binding peptide. EGF is a single-chain polypeptide of 53 residues with three disulfide bonds which define three looped regions 1-20, 14-31, and 32-53 (Savage *et al.*, 1972). It has been suggested that residues in positions 20-31 contain a major receptor binding region (Komoriya *et al.*, 1984) and that deletion of the COOH-terminal five or six residues leads to a marked reduction in receptor affinity (Cohen and Carpenter, 1975; Hollenberg and Gregory 1980). Due to its high degree of cross-species similarity, it is envisioned that any EGF receptor-binding protein from any

mammalian source can be employed according to the present invention.

While whole EGF is the preferred embodiment, it is believed that natural or chemically-synthesized fragments of EGF or other modified version of EGF that have significant EGF receptor-binding activity will function according to the present invention. These sequences are described further in the section on Biological Functional Equivalents, below.

The cognate receptor for EGF is over-expressed on the surface of lung tumor cell lines (Putnam *et al.*, 1992). A number of other tumor and normal cells also express an EGF receptor. While the examples provided herein address the use of EGF receptor-binding peptides to lung tumor cells, it is proposed that the invention is generally applicable to situations where an EGF peptide recognizes a receptor. In this regard, cancer cell types such as breast cancer, glioblastoma and squamous carcinoma are mentioned by way of example. In addition, normal tissue such as epithelium or muscle also provides a suitable target.

As mentioned above, EGF peptides need to retain some significant receptor binding activity in order to function according to the present invention. It is a routine matter to test for such binding with purified receptor *in vitro* by assays such as gel retardation assays, filter binding techniques, affinity chromatography and precipitation or sedimentation methods.

Alternatively, an *in situ* approach could be used. For example, one could label the peptide to be tested and mix the peptide with cells expressing the receptor on their cell surface under conditions which permit binding.

Subsequently, this mixture is submitted to treatment that separates the unbound peptide from bound peptide. The amount of peptide bound to cells can be determined and compared with a control samples comprising (i) peptide with no specific binding affinity for EGF receptor and (ii) cells lacking an EGF receptor. Further binding studies may be performed, including the use of EGF as a competitor, to compare affinities for receptor.

In yet another assay, functional output can be used to determine binding and cellular uptake of a complex via an EGF receptor. For example, a nucleic acid encoding a reporter gene can be conjugated to the EGF ligand, as discussed further below. When taken up by the appropriate cell, the reporter gene is expressed and the reporter product's activity monitored. One such reporter gene is β-galactosidase, as described in the disclosed example.

Nucleic Acid-Binding Moiety

Another element of the present invention is the nucleic acid-binding moiety. Nucleic acid-binding agents include proteins, polypeptides, peptides, antibodies, nucleotides, carbohydrates, fatty acids, organic or inorganic compounds or a combination of these and others. Nucleic acid-binding agents may bind to single-stranded or double-stranded DNA, to single-stranded or double-stranded RNA, by chemical or physical forces or by a combination of the two. A nucleic acid-binding agent may (i) have affinity only for the nucleic acid itself, (ii) have affinity for both the nucleic acid and another molecule, thereby forming a bridge between the two or (iii) have indirect affinity for the nucleic acid via affinity for another molecule that has affinity for the nucleic acid.

According to the present invention, the coupling of a nucleic acid-binding agent and EGF ligand must occur in such a manner that does not interfere with the binding of the EGF ligand to EGF receptor. Preferably, internalization of the EGF ligand complex via normal receptor-mediated endocytosis also is retained. In an even more preferred embodiment, this recognition and internalization delivers the nucleic acid into a target cell in a form suitable for the expression or for interaction with target endogenous nucleic acid.

In one embodiment, the nucleic acid-binding agent may insert itself between base pairs of double-stranded nucleic acids in an intercalative manner or bind in the minor or major groves of double-stranded nucleic acids. This binding may be sequence specific or completely unrelated to sequence. In other embodiments, nucleic acids may be cross-linked with other molecules with chemically of photochemically reactive groups.

In another embodiment of the invention, the nucleic acid-binding agent covalently links the nucleic acid to another molecule. In one embodiment the nucleic acid-binding agent is one of the coupling agent as described below such as carbodiimide. However, covalent coupling of the nucleic acid may alter its specificity and preclude proper gene expression or target nucleic acid recognition. Furthermore, linear or single stranded nucleic acid may be a requirement for covalent coupling of the nucleic acid to an EGF receptor. Finally, nucleic acids are negatively charged molecules which means that they may be repelled from cell surfaces, making transfer difficult via the endosomal lysis pathway. Therefore a size and type restriction may be necessary for the efficient delivery of nucleic acid directly bound to an EGF protein or fragment.

A preferred embodiment of the invention, that overcomes these problems, is a polycationic moiety that depends on electrostatic-dominated binding involving sequence-neutral interactions between the cationic groups and the negatively charged phosphates on nucleic acid. The polycationic moiety binds DNA strongly resulting in the formation of a toroid complex where the negative charge of nucleic acid molecule is completely neutralized. This soluble toroid complex may be internalized via normal receptor-mediated endocytosis. Any type of nucleic acid may be used, from single stranded mRNA to double stranded circular plasmids. Furthermore, any size of nucleic acid may be used, as long as there is a source of negative charge for the polycationic moiety to bind. In certain embodiments, these polycationic moieties, may include a natural polyamine such as spermine and spermidine. In a preferred embodiment, the polycationic moiety may be an artificially produced moiety, such as polylysine.

In order for the invention to function properly, certain criteria with regard to the nucleic acid-binding agent need to be fulfilled. First, the nucleic acid to be delivered into the cell must bind to the nucleic acid-binding agent without loosing its integrity in any way. Second, the complex comprising of ligand, nucleic acid-binding agent and nucleic acid must be in soluble form to allow greater accessibility of the complex to cells *in vitro* and *in vivo*. Third, once the complex is internalized within the host cell, the nucleic acid must have access to its target sequence while avoiding degradation.

Coupling of EGF Peptide and Nucleic Acid-Binding Moiety

As stated above, the complex includes an EGF moiety and a nucleic acid-binding moiety. The two moieties are

bound to each other via a linking entity. One example of such a linking entity is streptavidin (or avidin) and biotin. The streptavidin (or avidin)-biotin interaction is the strongest known non-covalent biological interaction between a protein and ligand (Pierce, 1994). Example 1, below, describes a method for complexing a ligand and nucleic acid-binding agent together by the streptavidin-biotin interaction. Streptavidin and biotin will first need to be coupled with the corresponding ligand or nucleic acid-binding agent. Any number of coupling agents may be used including those described below.

In another embodiment, the linking entity may include agents such as carbodiimides, N-succinimidyl,3-(2-pyridyldithio) propionate, succinimmidyl,4-(N-maleimidomethyl) cyclohexane-1-carboxylate, diisocyanates, glutaraldehyde, diazobenzenes, and hexamethylene diamines. This list is not intended to be exhaustive of the various coupling agents known in the art but, rather, is exemplary of the more common linking agents that may be used.

Endosomal Lysis Agent

In preferred embodiments, the present invention also includes an endosomal lysis agent that serves to increase release of the complex from endosomes once internalized by the cell but prior to fusion with a lysosome, thereby preventing degradation of the nucleic acid and/or the complex. An endosomal lysis agent is any agent that causes a disruption of the endosome sufficient to allow release of a complex without undue disruption of other cellular components. In one embodiment, adenoviral particles are an affective endosomal lysis agent. The particles may be mixed with the complex or coupled to the complex, as described in Example 1.

In preferred embodiments, modified adenoviruses are used to overcome potential safety problems. For example an infectious, replication-deficient adenovirus particle (Cotten *et al.*, 1992) or adenovirus with reduced toxicity (Cotten *et al.*, 1993) may be used. Other viruses, modified or unmodified, may be used in the present invention. These include, but are not limited to, human papilloma virus (HPV) or adeno-associated virus (AAV).

Alternatively, in still further embodiments, fusogenic viral peptides can be used in lieu of whole virus particles. For example, influenza hemagglutinin HA-2 terminal peptides have been demonstrated to augment gene transfer efficiency greater than 100-fold by the receptor-mediated gene transfer into HeLa cells (Wagner *et al.*, 1992; Plank *et al.*, 1994). In addition, recent studies in protein trafficking and endosomal physiology have identified several endogenous proteins associated with vesicle budding, membrane fusion (Rothman and Orci, 1992) and protein translocation (Gîrlich and Rapoport, 1993). These proteins can be used to reduce the lysosomal degradation of transgenes in receptor-mediated gene delivery.

Toxins, such as ricin, cholera enterotoxin, endotoxin, pertussis toxin, diphtheria toxin or fragments thereof may be employed as endosomal lysis agents. Toxins often have a high affinity for cell surfaces whereby they are introduced into the cell via the endosomal lysis system and then released. For example, ricin binds to galactose, and diphtheria toxin binds to ganglioside GM1, on cell surfaces. Unfortunately toxins are often harmful, if not lethal, to the cell. However, any modified, or fragment of, toxin, that maintains the ability to enter the cell and be released from the

endosome without undue damage to the cell may be used in the present invention.

Gene Delivery

The primary use for the complexes described above is in the delivery of genes to cells. Such delivery may be accomplished *in vitro*, as in laboratory procedures for transforming cells lines, or *in vivo*, as in the treatment of disease states for which a there is a genetic basis. Thus, it is proposed that the present invention is generally applicable to any situation where one desires high level expression of a recombinant protein in a target or host cell through the use of the invention.

Methods of gene transfer are commonly used in laboratories for a variety a different purposes - immortalization of cell lines, production of heterologous proteins, assays for protein function, cell tagging, *etc.* Thus, the compositions and methods described herein will be useful in standard laboratory procedures with a variety of different gene constructs. For example, standard expression cassettes including a gene of interest can be transferred to a target cell for transient expression. Other vectors that permit stable transformation, either chromosomal or episomal, may be used.

Another aspect of the invention concerns the binding of a therapeutic gene to an EGF receptor-binding peptide/nucleic acid binding agent. The term "therapeutic gene" is intended to refer to any foreign nucleic acid introduced into a cell for the potential benefit of the cell or to an organism containing that cell. This process may include the introduction of:

(i) a normal allele of a gene into a cell that either does not express its own copy of the gene or has a defective copy;

(ii) a normal or improved gene into a normal cell for the enhanced expression of a gene product;

(iii) suppressor genes to correct any endogenous mutations within the cell;

(iv) toxic genes, such as cytokines, to interfere with the expression of oncogenes or viral genes and thus inhibit neoplastic cell growth or viral replication; or

(v) antisense constructs and ribozymes that act on disease-related nucleic acid targets in cells.

While the nature of the gene introduced is not critical to the *in vitro* applications, it should be mentioned that in the context of cancer treatment modalities, a particularly useful gene is a tumor suppressor. There are numerous tumor suppressors well know to those in the art, preferred examples including p53, p16, RB, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC and MCC. This list is not intended to be exhaustive of the various tumor suppressors known in the art but, rather, is exemplary of the more common tumor suppressors.

A third modality for gene therapy involves the synthesis of antimicrobial agents within cells susceptible to infection by the corresponding viruses. In this context, the therapeutic gene could be any product that serves to block, reduce or inhibit viral replication and or damage to host cells.

In certain embodiments, the therapeutic gene is stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In further

embodiments, the gene may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle.

In preferred embodiments, the therapeutic gene would comprise complementary DNA (cDNA). The term "cDNA" used here, is intended to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic DNA or non- or partially-processed RNA template, is that the cDNA does not contain any non-coding sequences but, rather, encodes only the coding region of the corresponding protein. There may be times when the full or partial genomic sequence is preferred, however, such as where the non-coding regions are required for optimal expression.

In still further embodiments, the cDNA is operably linked to a promoter. Preferably, the promoter is active in one or more cell types expressing an EGF protein receptor and, more preferably, in a lung cell. A "promoter" refers to a DNA sequence recognized by the synthetic components of the cell, required to initiate the specific transcription of a gene. What is meant by the phrase "operably linked" is that the promoter is in the correct location and orientation in relation to the gene to allow the promoter to facilitate expression of the gene.

The particular promoter that is employed to control the expression of the therapeutic gene is not believed to be important, so long as it is capable of expressing the therapeutic gene in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the

therapeutic gene coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

The cytomegalovirus (CMV) promoter is a preferred promoter. One may also mention by way of example, and well known to those of the art, promoters derived from RSV, N2A, LN, or SV40. Preferred embodiments of the invention would include lung-specific promoters such as the Clara cell 10 kDa promoter (CC_{10}) (Stripp *et al.*, 1992), phosphoglycol kinase (PGK), or lung epithelial cell-specific surfactant protein B (SPB) (Bohinski *et al.*, 1994).

On the other hand, the therapeutic gene may be designed to alleviate a non-cancerous disease state. For example, cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Delivery of the CFTR gene into the lungs of affected individuals may prove to be an important therapy for CF. Furthermore, genetic therapy for hemophilia could be achieved by introduction of a therapeutic gene encoding a clotting factor into muscle cells.

In embodiments where inhibition or suppression of gene expression is desired, antisense molecules may be employed. The term "antisense nucleic acid" is intended to refer to the targeting of oligonucleotides against complementary base sequences in DNA and RNA. Extracellular oligonucleotides enter the cell and specifically bind their targets, interfering with transcription, RNA processing and transport or translation. Targeting double-stranded (ds) DNA with oligonucleotide leads to triple-helix formation; targeting RNA will lead to double-helix formation.

For certain applications, the antisense DNA or RNA that is introduced will be complementary to a selected cellular gene, such as an oncogene sequence or some other sequence whose expression one seeks to diminish through antisense application. While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs *in vitro* to determine whether cellular gene's function is affected or whether the expression of related genes having complementary sequences is affected.

In one embodiment, the antisense constructs of the present invention, whether antisense DNA molecules, antisense RNA molecules or DNA molecules which encode for antisense RNA molecules, will down-regulate oncogene expression. By preparing a construct that encodes an RNA or DNA molecule that is in antisense or "complementary" configuration with respect to the RNA or DNA of the target gene, the construct will inhibit or suppress the ultimate expression of the target gene, presumably by binding to the target RNA or DNA, and thereby preventing its translation or transcription, respectively.

The most preferred oncogenes for application of the present invention will be those which specifically exist in lung cells or any other cell that expresses an EGF protein receptor on its surface. One may mention, by way of example, oncogenes and oncogene families, such as *ras*, *myc*, *myb*, *mos*, *met*, *neu*, *raf*, *erb*, *src*, *fps*, *fms*, *jun* and *abl*. Again, this list is not intended to be exhaustive but, rather, is exemplary of the more common oncogenes.

In embodiments directed to infectious agents, with particular attention to agents that attack lung tissue, the antisense construct may correspond to a transcript for an organism such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, rhinovirus or coronavirus.

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to the an RNA-based enzyme capable targeting cleaving particular base sequences in DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as DNA encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense nucleic acids.

Biological Functional Equivalents

As mentioned above, changes may be made in the structure of EGF receptor-binding peptide while maintaining the desirable receptor-binding characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites of ligands such as

an EGF receptor-binding peptide. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. Equally, the same considerations may be employed to create a protein or polypeptide with countervailing (e.g., antagonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of an EGF receptor-binding peptide (or underlying DNA) without appreciable loss of their biological utility or activity.

It is also well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. It also is well understood that where certain residues are shown to be particularly important to the biological or structural properties of a protein or peptide, *e.g.*, residues in active sites, such residues may not generally be exchanged.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these

considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

To effect more quantitative changes the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine

(+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

While discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA, taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid.

Structural Functional Equivalents and Sterically Similar Constructs

In addition to the peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modelling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

Pharmaceutical Compositions

Where clinical application of EGF receptor-binding peptide/nucleic acid-binding agent is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the nucleic acid, such as a therapeutic gene, bound to an EGF receptor-binding peptide/nucleic acid binding agent, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions can also be referred to as inocula. As mentioned above, therapeutic gene is intended to refer to any nucleic acid introduced into a cell for the potential benefit of the cell or the individual organism as a whole. This includes, among other things, as obvious to one skilled in the art, tumor suppressors, cytokines, antisense constructs and ribozymes.

The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for

pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

In addition to the compounds formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, *e.g.*: inhalents and such like, that administer the active ingredients by aerolization; tablets or other solids for oral administration; time release capsules; and any other form currently used, including cremes, lotions and even mouthwashes.

The active compounds also may be formulated for parenteral administration, *e.g.*, formulated for injection via the intravenous, intramuscular, subcutaneous, intratracheal or even intraperitoneal routes. The preparation of an aqueous composition that contains the therapeutic gene complex as an active ingredient will be known to those of skill in the art in light of the present disclosure. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for using to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and the preparations can also be emulsified.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of

storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

A therapeutic gene complexed to an EGF receptor-binding peptide/nucleic acid-binding agent of the present invention, can be formulated into a composition in a neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

Therapeutic formulations in accordance with the present invention may also be reconstituted in the form of inhalants which may contain EGF receptor-binding peptide/nucleic acid binding agent and therapeutic gene complex alone, or in conjunction with other agents, such as, *e.g.*, pentamidine.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial ad antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus ny additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more or highly concentrated solutions for intramuscular injection is also

contemplated. In this regard, the use of DMSO as solvent is preferred as this will result in extremely rapid penetration, delivering high concentrations of the active peptide, peptides or agents to a small area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

Therapeutic Kit Components

Therapeutic kits comprising an EGF receptor-binding peptide and a nucleic acid-binding agent form another

aspect of the invention. Such kits will generally contain, in suitable container means, pharmaceutically-acceptable formulation of the complex comprising of EGF receptor-binding peptide and a nucleic acid-binding agent. Also included in the kit may a nucleic acid of choice and a pharmaceutically-acceptable formulation of an endosomal lysis agent. The kit may have a single container means that contains EGF receptor-binding peptide and nucleic acid agent, with or without the nucleic acid of choice and the endosomal lysis agent, or it may have distinct container means for each compound.

When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred. The EGF receptor-binding peptide/nucleic acid agent may also be formulated into a syringeable composition. In this case, the container means may itself be an inhalent, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means.

The container means generally will include at least one vial, test tube, flask, bottle, syringe or other container means, into which an EGF receptor-binding peptide/nucleic acid agent/nucleic acid complex may be placed, The kit also will generally contain a second vial or other container into which the endosomal lysis

agent may be placed. The kits also may comprise a second/third container means for containing a sterile, pharmaceutically acceptable buffer or other diluent.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired vials are retained.

Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalent, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

Targeted Nucleic Acid Delivery to Cells

In order for an EGF peptide/nucleic acid-binding agent complex to deliver nucleic acid to a cell, it must first contact the cell. The phrase "contact," as used herein, is intended to refer to the structural proximity of two or more moieties needed before functional interaction can occur. In the present invention, functional interaction may be defined as the recognition of the complex by EGF receptor on the target cell surface and subsequent internalization of the complex by the cell. In order to contact an EGF peptide/nucleic acid-binding agent complex with a cell *in vitro*, it is a simple matter to add or admix the complex with the cells, as evidenced in Example 1.

In the *in vivo* setting, contacting the target cell or tissue could involve direct injection or inhalation.

Alternatively, it could involve the administration of the soluble EGF complex into the blood stream. Size may play a role in successful delivery and contact of the complex to the relevant cells. Previous study of an ASOR-polylysine-DNA complex prepared under similar procedures gave molecular size of 80-100 nm (Cristiano *et al.*, 1993). Perales *et al.* (1994) have recently reported that by changing salt conditions, it is possible to modulate the sizes of galactosylated poly(L-lysine)/DNA complex (to about 10 nm). The formation of such small complexes was found to correlate with the prolonged expression of transgene in the livers of intact animals.

Another *in vitro* use of the present invention is to determine whether cells or cell lines, express functional EGF receptor on their surface. This can be evaluated by admixing the EGF ligand/nucleic acid-binding agent complexed with a reporter gene with the cells to be evaluated, as described in Example 1. Where the reporter gene is β-galactosidase, cells expressing or overexpressing EGF receptor will produce clearly visible, blue-stained cells. This procedure may be used for diagnosing cancer cells as many tumor cells overexpress the EGF receptor. This would be particularly valuable for lung, breast and glioblastoma cells.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the example which follows represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result

without departing from the spirit and scope of the invention.

EXAMPLE I

A. Materials and Methods

1. *EGF/PLL/DNA complex Formation*

Recombinant human EGF (Biosource Intl., Camarillo, Ca.) was coupled to poly-L-lysine (PLL) (Molecular Weight, 22,500; Sigma Chemical Co., St. Louis, Mo.) in a three step procedure. First, EGF was biotinylated with biotin hydroxysuccinimide ester (Pierce Chemical Co., Rockford, IL) (Kayser *et al.*, 1990), yielding one biotin per EGF molecule. The unreacted biotin ester was removed by dialysis in HBS (150 mM NaCl, 20 mM HEPES, pH 7.3) by using a 1000 MWCO membrane (Fisher Scientific, Pittsburg, Pa.). The second step involved the introduction of a disulfide bond between streptavidin and PLL. Streptavidin (Pierce Chemical Co.) was modified with the use of N-Succinimidyl-3-(2-pyridyldithio)-propionate (Pierce Chemical Co.) to contain one sulfhydryl group (Carlsson *et al.*, 1972). Poly-L-lysine was modified to contain one sulfhydryl group by using Traut's reagent (Pierce Chemical Co.) (Jue *et al.*, 1978). The modified streptavidin and PLL were purified from unreacted components by using a G-25 sephadex column.

The modified streptavidin and modified PLL were then combined and allowed to incubate overnight at room temperature under argon, to allow for disulfide bond formation. The resulting streptavidin/PLL was then purified by using a Mono Q column (Pharmacia Fine Chemicals, Piscataway, NJ.). The final sample contained streptavidin linked to PLL in a one-to-one ratio. The

third step involved incubation of the EGF/biotin with the streptavidin/PLL for 30 min. at room temperature.

The DNA/protein complexes were made by adding increasing molar ratios of the EGF/PLL conjugate in 150 μl of HBS to 6 μg of DNA in 350 μl of HBS, vortexing the sample and then incubating for 30 min. at room temperature. The resulting samples were either analyzed by agarose gel electrophoresis to determine charge neutralization on the DNA sample or added to cells for uptake studies. The plasmid pCMV/β-Gal, which contains the *E. coli* beta-galactosidase (β-Gal) gene under the control of the cytomegalovirus enhancer and promoter, was used for cell transduction studies. All plasmid DNA preparations were purified by using the Mega Prep plasmid preparation kit (Qiagen, Studio City, Ca). A diagrammatic representation of the EGF/PLL/DNA complex can be seen in FIG. 1.

2. *Adenovirus Isolation and Modification*

A replication defective adenovirus, which does not express E1A, was used as an endosomal lysis agent. The adenovirus (ADV) was grown in 293 cells (a human embryonic kidney cell line) and purified by the following protocol (Cristiano *et al.*, 1993). ADV was purified by double banding on CsCl gradients (Massie *et al.*, 1986) and then dialyzed against 2x filtered Hepes-buffered saline (HBS = 150 mM NaCl/20 mM Hepes·NaOH, pH 7.3). The concentration of the virus was determined by UV-spectrophotometric analysis and either stored in 10% (vol/vol) glycerol at - 20°C or further modified for DNA complex formation.

For modification, freshly isolated ADV (1.4×10^{11} particles) was combined with PLL (Sigma, 20.5 kDa) at 16 μM, along with 1-ethyl-3-(3-dimethylaminopropyl)

carbodiimide (EDC) at a final concentration of 130 μM (low EDC) or 2.6 mM (high EDC) in 4 ml. After incubation on ice for 4 h, the unreacted components were removed by ultracentrifugation (150,000 x g) for 18 h on a CsCl gradient at a CsCl concentration of 1.35 g/ml. The ADV (E5) was then either dialyzed against 2 M NaCl and then stored at - 20°C in 10% glycerol or stored in 10% glycerol (Cristiano *et al.*, 1993). A diagrammatic representation of the EGF/PLL/DNA complex coupled to adenovirus can be seen in FIG. 2.

3. *Cell Culture and EGF/PLL/DNA complex Analysis*

All incubations of the EGF/PLL/DNA complex, adenovirus, or DNA/modified adenovirus complexes with the cells were done in 1 ml of basic cell culture medium with 2% fetal calf serum for 2 h at 37°C in a CO_2 incubator. Following the incubation, 2 ml of complete medium was added back to the cells, which were then incubated for 24 h at 37°C in a CO_2 incubator. β-Gal expression was analyzed by either histologic staining or quantitated through an 0-Nitrophenyl β-D-Galactopyranoside based assay according to published protocols (Nielson *et al.*, 1983).

The cell lines used in the analysis were SW620, a colon adenocarcinoma (Murphy *et al.*, 1990); H460a (Putnam *et al.*, 1992) and H1299 (Mitsudomi *et al.*, 1992), large cell lung carcinomas; H322 (Mitsudomi *et al.*, 1992), H226b (Putnam *et al.*, 1992), and H226Br (Fujiwara *et al.*, 1994), squamous lung carcinomas; H596 (Mitsudomi *et al.*, 1992), an adenosquamous lung carcinoma; and H358 (Mitsudomi *et al.*, 1992), a broncho-alveolar cell lung carcinoma. All cell lines were plated in 6-well plates (Falcon Plastics, Oxnard, Ca.) in the appropriate medium 48 h prior to incubation in quantities sufficient to

result in a density of $5x10^5$ cells per well at the time of use.

B. Results

1. *EGF/PLL/DNA complex Formation*

To determine whether the EGF/PLL conjugate was capable of binding DNA, increasing molar amounts of the EGF/PLL conjugate were incubated with a set amount of the plasmid pCMV/β-Gal. The samples were then analyzed by gel electrophoresis on a 1% agarose gel. Correct binding of the EGF/PLL conjugate with the DNA should result in the charge on the DNA being neutralized, causing it to be retained in the well of the gel. This charge neutralization has been shown to contribute to efficient DNA delivery into cells (Cristiano *et al.*, 1993). When no EGF/PLL or EGF/biotin without streptavidin/PLL was incubated with the DNA, the charge on the DNA did not neutralize. As the amount of the EGF/PLL conjugate was increased, a greater proportion of the DNA was rendered neutral, with the DNA being completely neutralized at a molar ratio of EGF/PLL to DNA of 150/1.

2. *Analysis of the EGF/PLL/DNA complex Uptake into Cells*

The EGF/PLL/DNA complex that showed complete charge neutralization was then incubated with three different cell lines; SW620, H460a, and H1299. The cell line SW620 does not express EGF receptors at the cell surface (Murphy *et al.*, 1990). The cell line H460a has been determined to have $6x10^4$ EGF receptors per cell and a higher affinity for EGF (Putnam *et al.*, 1992). The status of the EGF receptor on the cell line H1299 has not been determined. H460a has a mutation in the *K-ras* gene and H1299 has a deletion of the *p53* gene. When 3 μg of

the plasmid pCMV/β-Gal in complex form with the EGF/PLL conjugate was incubated with each cell type, none of the cells showed staining indicative of β-Gal expression.

To determine whether the adenovirus enhanced the delivery of the EGF/PLL/DNA complex into the cell through endosomal lysis, 3 μg of the EGF/PLL/DNA complex was added to the cells along with an increasing amount of the adenovirus (FIG. 3). At a viral titer of 10^2 viral particles/cell, there was a slight increase in the percentage of H460a and H1299 cells staining positive. The maximum number of positive-staining cells for these cell lines occurred at a concentration of $3x10^3$ adenoviral particles/cell, whereas the cell line SW620 showed very low numbers of cells staining positive for β-Gal activity at all adenoviral titers used.

To further analyze the specificity of uptake, the cells were incubated with 3 μg of DNA in complex form along with 10^3 adenoviral particles/cell and with a 200-fold molar excess of free EGF. The percentage of positive staining cells was reduced from 65% to 12% for the H460 cell line and reduced from 35% to 8% for the H1299 cell line, indicating specific delivery through the EGF receptor. When competition was done with a 200-fold molar excess of insulin, no reduction in positive staining cells occurred.

Although the EGF/PLL/DNA complex can be delivered efficiently into lung cancer cell lines along with the help of the replication-defective adenovirus, a more suitable complex for DNA delivery *in vitro* and *in vivo* would be the direct coupling of the adenovirus to the complex. This has also been shown to be effective with other DNA/protein complexes (Cristiano *et al.*, 1993).

To test this type of complex on the lung cancer cell lines, a virus that was modified to have a large amount of PLL attached to the viral capsid was generated. This modified adenovirus (E5) has been shown to have a large proportion of targeting occur through the coupled ligand and its receptor (Cristiano *et al.*, 1993). When DNA was delivered by coupling the EGF/PLL/DNA complex to E5, the efficiency of DNA delivery into the cells was enhanced (FIG. 4).

The titer of the adenovirus necessary to achieve maximal transduction was between $3x10^2$ and 10^3 adenoviral particles/cell, almost an order of magnitude less than was needed when the complex was simply administered along with the unmodified adenovirus. This was seen by levels of transduction in the cell lines H460a and H1299 (FIG. 4). The uptake of the EGF/DNA/E5 complex by the cell line SW620 was also increased (up to 15% transduction), possibly indicating that the EGF/PLL/DNA complex has a greater ability to enter the cell through the adenoviral receptor when it is linked to the adenovirus.

A similar competition analysis was done using the EGF/DNA/E5 complex at 10^3 adenoviral particles/cell along with a 200-fold molar excess of free EGF. The percentage of positive staining cells was reduced from 90% to 60% for the H460 cell line and reduced from 99% to 67% for the H1299 cell line, indicating that a large percentage of DNA delivery was occurring through the EGF receptor. When competition was done with a 200-fold molar excess of insulin, no reduction in positive staining cells occurred.

3. *Quantitation of Beta-Galactosidase Expression*

To determine whether the type of complex had an effect on overall levels of gene expression, the β-Gal expression was quantitated. When 3 μg of pCMV/β-Gal in complex form was incubated with the cells along with 10^3 adenoviral particles/cell, the levels of β-Gal expression in the cell lines H1299 and H460a ranged from 0.7 to 0.9 units of β-Gal/5x10^5 cells, whereas no β-Gal was detected in the cell line SW620 (FIG. 5), which is permissive for adenovirus infection. This indicates that the DNA/protein complex was being taken up through the EGF receptor and not through the adenovirus receptor.

When the EGF/PLL/DNA complex was coupled to the modified adenovirus E5, the level of β-Gal expression was greatly increased, with up to 4.4 units of β-Gal/5x10^5 cells expressed (FIG. 5). The cell line SW620 also showed an increase in β-Gal expression when the modified adenovirus was used, again indicating that a small percentage of the DNA was being delivered through the adenovirus receptor, but at much lower levels than the amount that entered through the EGF receptor.

4. *DNA Delivery into lung Cancer Cell Lines*

The ability of the EGF/PLL/DNA complexes to deliver DNA into several different lung cancer cell lines was assessed. The complex, along with the adenovirus (either non-modified or modified), was incubated with the cells at a viral titer of 10^3 adenoviral particles/cell (FIG. 6). When the complex was used with the non-modified adenovirus, the levels of transduction ranged from 15% (H226b) to 70% (H460a) (FIG. 6). When the E5 adenovirus was used along with the EGF/PLL/DNA complex, the percentage of cells transduced ranged from 15% (H226b) to 99% (H1299) (FIG. 6). In each cell line

tested, the coupling of the adenovirus to the EGF/PLL/DNA complex enhanced the delivery of the DNA into the cells.

The EGF/PLL/DNA complex allowed the efficient delivery of DNA into several lung cancer cell lines. The results shown here agree with other reports in which the attachment of DNA to protein/PLL conjugates resulted in a receptor-dependent process for uptake that could be enhanced by an endosomal lysis agent.

The uptake of the EGF/PLL/DNA complex occurred specifically through the EGF receptor, which was shown by the lack of DNA uptake into the cell line SW620 and by competition with free EGF, when analyzed by either histologic staining or quantitation of β-Gal expression.

The delivery of DNA by the complex was enhanced by adenovirus, which acts as an endosomal lysis agent. The efficiency of delivery was further enhanced by coupling the complex directly to the adenovirus as seen by the increase in the number of cells transduced when the E5 modified adenovirus was used.

The delivery of DNA by the EGF/DNA/E5 complex could be competed with excess free EGF, but this was not as successful as when the complex and the adenovirus were separate. This may be due to the following factors; 1) the interaction of the adenovirus with its receptor, as seen by an increase in the number of cells staining positive for the cell line SW620, 2) a change in the interaction between the EGF and its receptor, as there are more EGF molecules per EGF/DNA/E5 complex, 3) the larger number of DNA molecules that are being delivered by the complex, contributing to higher levels of expression being achieved, or 4) a combination of the above factors.

Although all the cell lines except SW620 showed a greater efficiency of DNA delivery when the DNA was attached to the adenovirus, there was variability in the levels of transduction among cell lines and also by the type of complex used. This was probably due to several factors: 1) differences in the number of EGF receptors expressed by each cell line; 2) differences in the ability of the adenovirus to either infect the cells or cause endosomal lysis in these cells; and 3) differences in receptor affinity for EGF, which is known to be higher for some cell types, such as H460a (Putnam *et al.*, 1992).

Based upon these studies, one of skill in the art, would expect that this system would allow for the delivery of therapeutic genes to lung tumors and other solid tumors that overexpress the EGF receptor. Since the EGF receptor is expressed on many different benign cell types, the fact that it is overexpressed on lung cancer cells may provide selectivity for the EGF/PLL/DNA complex. Other levels of specificity could be generated by using lung tumor-cell specific promoters as well as specific types of therapeutic genes.

* * *

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art

are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

Bohinski *et al.* The lung-specific surfactant protein B gene promoter is a target for thyroid transcription factor 1 and hepatocyte nuclear factor 3, indicating common factors for organ-specific gene expression along the foregut axis. *Mol. Cell. Biol.* **14**(9):5671-5681 (1994).

Carlsson, J., Drevin, H., and Axen, R. Protein thiolation and reversible protein-protein conjugation. *Biochem. J.* **173**:723-737 (1972).

Ceremi, C., Frevert, U., Sinnis, P. Takacs, B., Clavijo, P., Santos, M.J. and Nussenzweig, V. *Cell* **70**:1021-1033 (1992).

Cohen and Carpenter. *Proc. Natl. Acad. Sci. USA* **72**:1317-1321 (1975).

Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D.T., and Birnstiel, M.L. *Proc. Nat'l Acad. Sci. USA* **89**:6094-6098 (1992).

Cotten, M., Wagner, E., Zatloukal, K., and Birnstiel, M.L. *J. Virol.* **67**:3777-3785 (1993).

Cristiano, R.J., Smith, L., and Woo, S.L.C. Hepatic gene therapy: efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus-DNA complex. *Proc. Nat'l Acad. Sci. USA* **90**:11548-11552 (1993).

Cristiano, R., Smith, L.C., and Woo, S.L.C. *Proc. Nat'l Acad Sci. USA* **90**:2122-2126 (1993a).

European Patent Application No. 0,273,085, published July 6, 1988.

Ferkol *et al.* *FASEB J.* **7**:1081-1091 (1993).

Fujiwara, T., Cai, D.W., Georges, R.N., Mukhopadhyay, T., Grimm, E.A., and Roth, J.A. Therapeutic effect of a retroviral wild-type *p53* expression vector in an orthotopic lung cancer model. *J. Nat'l Cancer Instit.* **86**:1458-1462 (1994).

Gîrlich, D. and Rapoport, T.A. *Cell* **75**:615-630 (1993).

Gottschalk, S., Cristiano, R.J., Smith, L., and Woo, S.L.C. Folate-mediated gene delivery and expression *in vitro*. *Gene Ther.* **1**:185-191 (1994).

Goundis, D. and Reid, K.B.M. *Nature* **335**:82-85 (1988).

Hammar, S.P. *In*: *Pulmonary Pathology*, pp. 727-845 (S.P. Hammar ed.), Springer Verlag Press, NY (1988).

Hollenberg and Gregory. *Mol. Pharmacol.* **17**:314-320 (1980).

Jue, R., Lambert, J.M., Pierce, L.R., and Traut, R.R. Addition of sulfhydryl groups to *Escherichia coli* ribosomes by protein modification with 2-iminothiolane (methyl 4-mercaptobutyrimidate). *Biochem.* **17**:5399-5405 (1978).

Kayser, K., Weisse, G., Gabius, H.J., and Hintze, T. Biotinylated epidermal growth factor: a useful tool

for the histochemical analysis of specific binding sites. *Histochem. J.* **22**:426-432 (1990).

Komoriya *et al.* Biologically active synthetic fragments of epidermal growth factor: Localization of a major receptor-binding region. *Proc. Natl. Acad. Sci. USA* **81**:1351-1355 (1984).

Kyte, and Doolittle, *J. Molec. Biol.* **157**:105-132 (1982).

Massie *et al.* *Mol. Cell. Biol.* **6**:2872-2883 (1986).

Miller, A.D. Human gene therapy comes of age. *Nature* **357**:455-460 (1992).

Mitsudomi, T., Steinberg, S. M., Nau, M. M., Carbone, D., D'Amico, D., Boder, S., Oie, H. K., Linnoila, R. I., Mulshine, J. L., Minna, J. D., and Gazdar, A. F. *p53* gene mutations in non-small-cell lung cancer cell lines and their correlation with the presence of *ras* mutations and clinical features. *Oncogene* **7**:171-180 (1992).

Morgan and Anderson. *Ann Rev. Biochem.* **62**:191-217 (1993).

Mulligan, R.C. The basic Science of gene therapy. *Science* **260**:926-931 (1993).

Murphy, L. D., Valverius, E. M., Tsokos, M., Mickley, L. A., Rosen, N., and Bates, S. E. Modulation of EGF receptor expression by differentiating agents in human colon carcinoma cell lines. *Cancer Comm.* **2**:345-355 (1990).

Neilsen *et al.* *Proc. Natl. Acad. Sci. USA* **80**:5198-5202 (1983).

Perales, J.C., Ferkol, T., Beegen, H., Ratnoff, O.D., and Hanson, R.W. *Proc. Nat'l Acad. Sci. USA* **91**:4086-4090 (1994).

Pierce Chemical Company catalog (1994), Pierce Chemical Company, P.O. Box 117, Rockford, IL.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. *J. Biol Chem.* **269**:12918-12924 (1994).

Putnam, E.A., Yen, N., Gallick, G.E., Steck, P.A., Fang, K., Akpakip, B., Gazdar, A.F., and Roth, J.A. Autocrine growth stimulation by transforming growth factor-α in human non-small cell lung cancer. *Surg. Oncol.* **1**:49-60 (1992).

Remington's Pharmaceutical Sciences, 15th ed., pp. 1035-1038 and 1570-1580.

Rich, K.A., George IV, F.W., Law, J.L., and Martin, W.J. *Science* **249**:1574-1577 (1990).

Robson, K.J.H., Hall, J.R.S., Jennings, M.W., Harris, T.J.R., Marsh, K., Newbold, C.I., Tate, V.E., and Weatherall, D.J. *Nature* **335**:79-82 (1988).

Rothman, J.E., and Orci, L. *Nature* **355**:409-415 (1992).

Savage *et al.* *J. Biol. Chem.* **247**:7612-7621 (1972).

Stripp *et al.* *cis*-Acting Elements That Confer Lung Epithelial Cell Expression of the CC_{10} Gene. *J. Biol. Chem.* **267**(21):14703-14712 (1992).

Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M.L. Transferrin-polycation conjugates

as carriers for DNA uptake into cells. *Proc. Nat'l Acad. Sci. USA* **87**:3410-3414 (1990).

Wagner *et al.* *Proc. Natl. Acad. Sci. USA* **89**:7934-7938 (1992).

Wang *et al.* *J. Biol. Chem.* **269**:9137-9146 (1994).

Wu and Wu. *J. Biol. Chem.* **262**:4429-4432 (1987).

Wu, G.Y., Wilson, J. M., Shalaby, F., Grossman, M., Shafritz, D. A., and Wu, C.H. Receptor-mediated gene delivery *in vivo*. *J. Biol. Chem.* **266**:14338-14342 (1991).

Wu and Wu. *Adv. Drug Delivery Rev.* **12**:159-167 (1993).

CLAIMS:

1. A complex comprising:

(i) a ligand comprising an EGF receptor-binding peptide; and

(ii) a nucleic acid-binding agent.

2. The complex of claim 1, wherein said peptide is EGF.

3. The complex of claim 2, wherein said peptide is a subfragment of EGF.

4. The complex of claim 1, wherein said nucleic acid-binding agent is a polycationic moiety.

5. The complex of claim 4, wherein said nucleic acid-binding agent is polylysine.

6. The complex of claim 1, further comprising an endosomal lysis agent.

7. The complex of claim 6, wherein said endosomal lysis agent is an infectious, replication-deficient adenovirus.

8. The complex of claim 1, further comprising a nucleic acid.

9. The complex of claim 8,, wherein said nucleic acid encodes a therapeutic gene.

10. The complex of claim 9, wherein said therapeutic gene is a cDNA.

11. The complex of claim 10, wherein said therapeutic gene further comprises a promoter that is active in a cell expressing EGF receptor and operably linked to said gene.

12. The complex of claim 11, wherein said gene encodes a tumor suppressor.

13. The complex of claim 12, wherein said tumor suppressor corresponds to *p53*.

14. The complex of claim 12, wherein said tumor suppressor corresponds to *p16*.

15. The complex of claim 11, wherein said gene encodes a cystic fibrosis transmembrane conductance regulator (CFTR).

16. The complex of claim 11, wherein said gene encodes blood clotting factor IX.

17. The complex of claim 1, further comprising a nucleic acid encoding an antisense construct or ribozyme.

18. The complex of claim 17, wherein said antisense construct or ribozyme corresponds to an oncogene transcript.

19. The complex of claim 18, wherein said oncogene transcript corresponds to *ras*.

20. The complex of claim 8, wherein said nucleic acid is at least of a length selected from the group consisting of 10, 20, 50, 100, 500, 1000, 5000 and 10,000 base pairs.

21. A pharmaceutical composition comprising:

(i) a complex comprising

(a) a ligand comprising an EGF receptor-binding peptide,

(b) a nucleic acid-binding agent, and

(c) a nucleic acid comprising said therapeutic gene; and

(ii) a pharmaceutically-acceptable carrier, diluent or excipient.

22. A kit comprising:

(i) a ligand comprising an EGF receptor-binding peptide; and

(ii) a nucleic acid-binding agent.

23. The kit of claim 22, further comprising nucleic acid encoding a nucleic acid.

24. The kit of claim 22, wherein said ligand and said nucleic acid-binding agent are complexed.

25. The kit of claim 23, wherein said ligand, said nucleic acid-binding agent and said nucleic acid are complexed.

26. A method of delivering a gene to a cell expressing EGF receptor comprising the steps of:

(i) providing a complex for the delivery of a gene to a cell comprising

(a) a ligand comprising an EGF receptor-binding peptide,

(b) a nucleic acid-binding agent, and

(c) a nucleic acid comprising said gene; and

(ii) contacting said complex with said cell.

27. The method of claim 26, wherein step (ii) further comprises contacting said cell with an endosomal lysis agent.

28. The method of claim 27, wherein said endosomal lysis agent is an infectious, replication-deficient adenovirus.

29. The method of claim 26, wherein said gene is a cDNA.

30. A method of treating a mammal with a lung tumor *in vivo* comprising:

(i) providing a pharmaceutical composition comprising

(a) a complex for the delivery of a therapeutic gene to lung cancer cell comprising

(1) a ligand comprising an EGF receptor-binding peptide,

(2) a nucleic acid-binding agent, and

(3) a nucleic acid comprising said therapeutic gene; and

(b) a pharmaceutically-acceptable carrier, diluent or excipient; and

(ii) administering said pharmaceutical composition to said mammal.

31. The method of claim 30, wherein said cells of said lung tumor express EGF receptor.

32. The method of claim 30, wherein said therapeutic gene is an antisense construct or ribozyme.

33. The method of claim 30, wherein said pharmaceutical composition further comprises an endosomal lysis agent.

34. The method of claim 33, wherein said endosomal lysis agent is an infectious, replication deficient adenovirus.

35. The method of claim 30, wherein said administering is selected from the group consisting of intravenous injection, subcutaneous injection, direct perfusion, inhalation, intra-tracheal injection and intra-peritoneal injection.

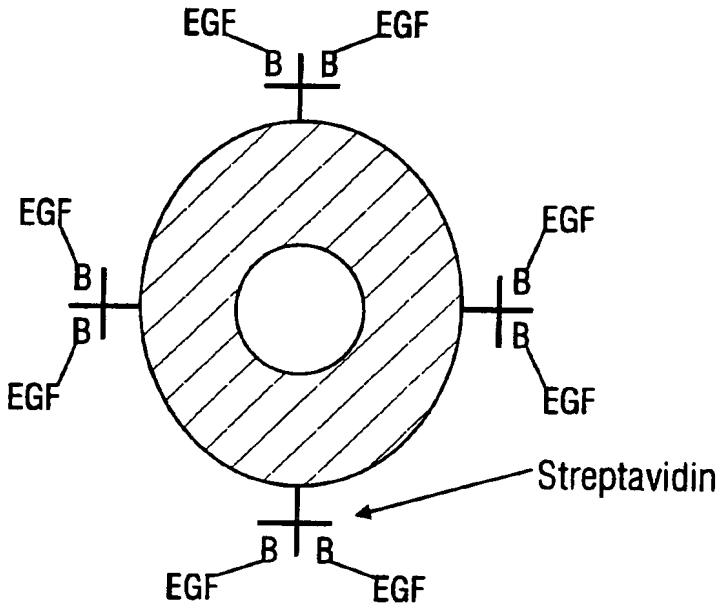


Fig. 1

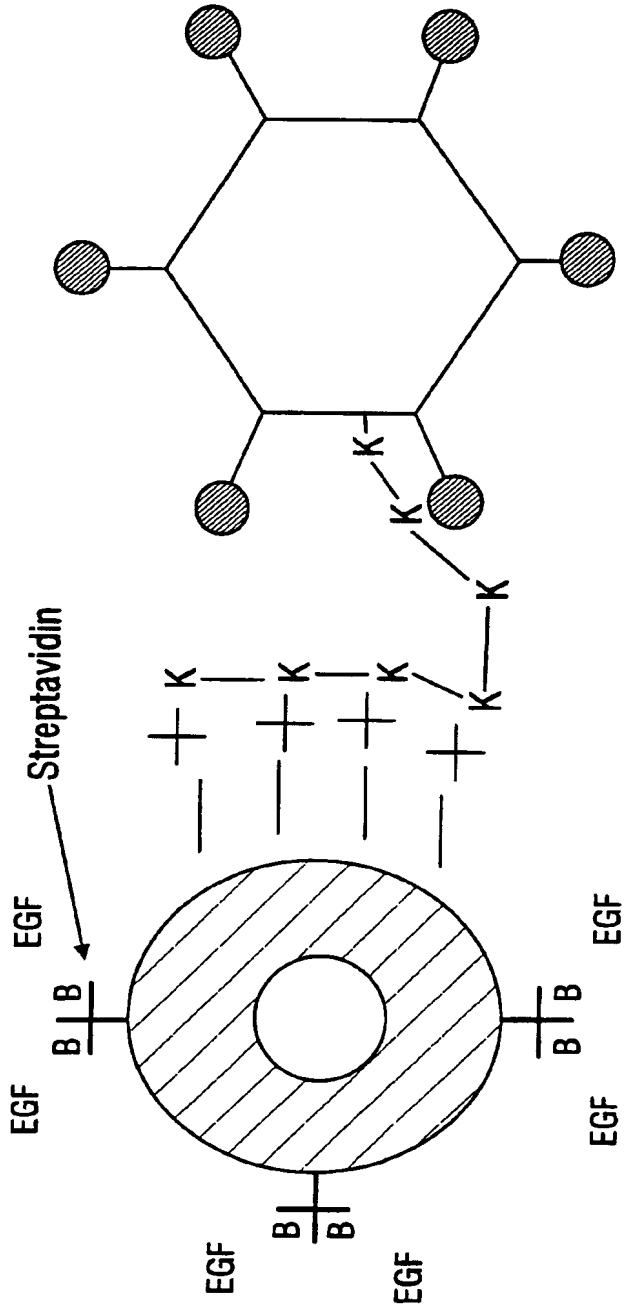
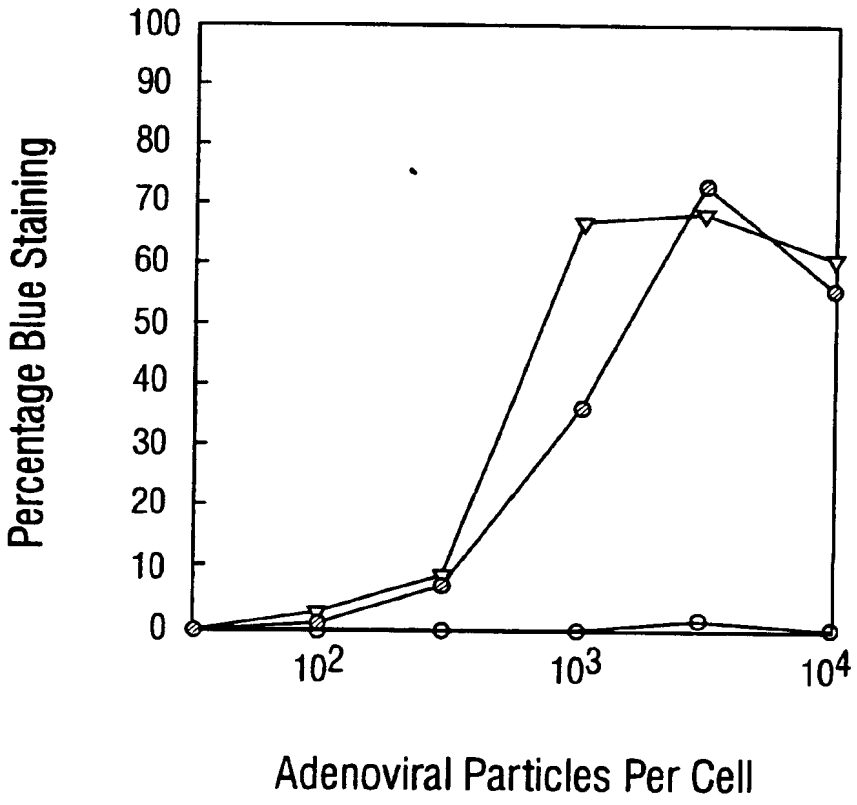


Fig. 2


Percentage Blue Staining
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80
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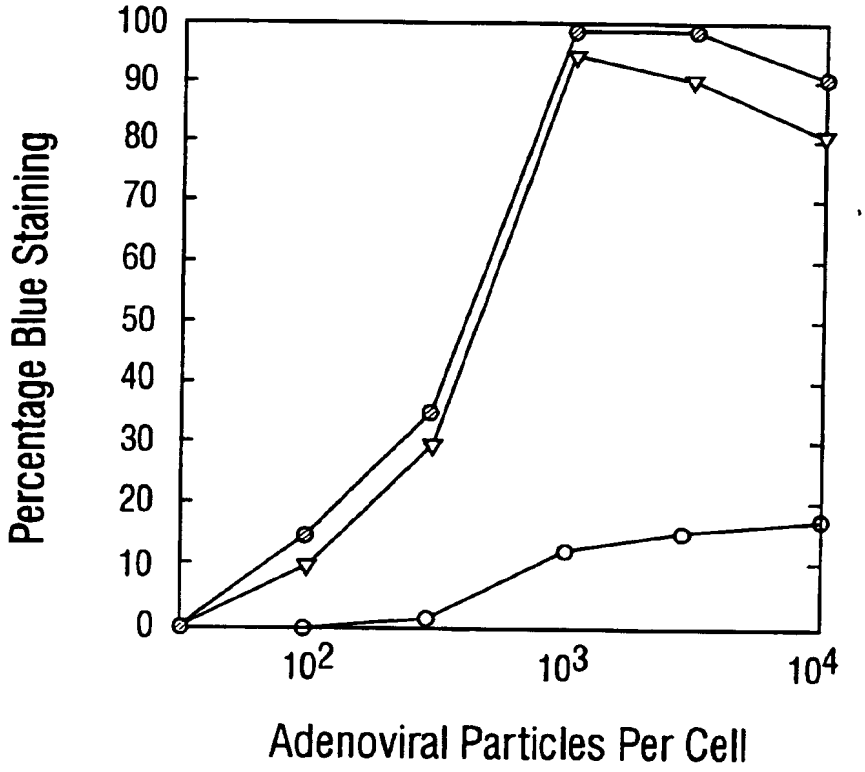
50
40
30
20
10
0
10^2
10^3
10^4
Adenoviral Particles Per Cell

Fig. 3


Percentage Blue Staining
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10
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10^2
10^3
10^4
Adenoviral Particles Per Cell

Fig. 4

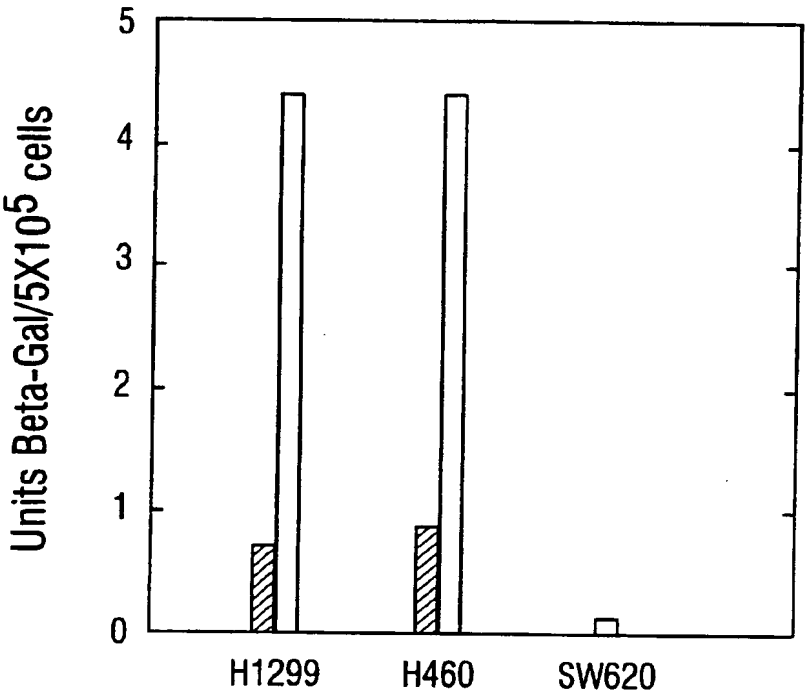
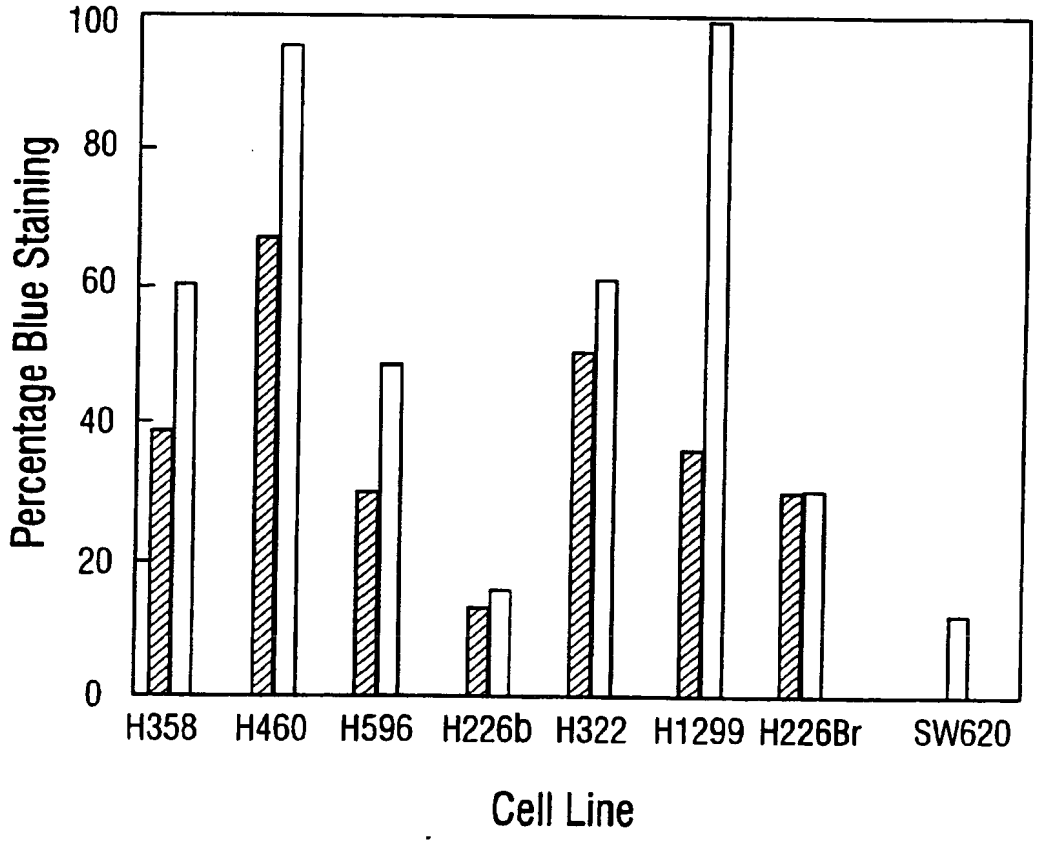


Fig. 5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80
60
40
20
0
Percentage Blue Staining
H358
H460
H596
H226b
H322
H1299
H226Br
SW620
Cell Line

Fig. 6

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PC1/US 96/04017

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87 C07K14/485 A61K38/18 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FEBS LETTERS, vol. 338, no. 2, 31 January 1994, AMSTERDAM NL, pages 167-169, XP002009636 J.CHEN ET AL.: "A novel gene delivery system using EGF receptor-mediated endocytosis" see the whole document --- -/--	1,4,5, 8-12, 20-26, 29-31

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
30 July 1996	09. 08. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Cupido, M

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 96/04017

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPLEMENT 17D, 1993, page 129 XP002009637 K.ZATLOUKAL ET AL.: "Receptor-mediated cytokine delivery to tumor cells for generation of cancer vaccines. Keystone symposium on cellular immunity and the immunotherapy of cancer, Taos New Mexico, USA March 17-24, 1993" see abstract NZ 522	1,4-12, 20-31
X	EP,A,0 273 085 (BATELLE MEMORIAL INSTITUTE) 6 July 1988 cited in the application see the whole document	1-3, 8-12, 19-26
X	WO,A,91 14696 (GILEAD SCIENCES, INC.) 3 October 1991 see claim 11	1-3,8-10
P,X	CANCER GENE THERAPY, vol. 3, no. 1, January 1996, pages 4-10, XP000577163 R.J.CHRISTIANO AND J.A.ROTH: "Epidermal growth factor mediated DNA delivery into lung cancer cells via the epidermal growth factor receptor" see the whole document	1-35

1

INTERNATIONAL SEARCH REPORT

Inte ional application No.
PCT/US96/04017

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US96/04017

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Although claims 26-29 insofar as in vivo method is concerned and claims 30-35 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/ composition.

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Intern· ·nal Application No
PCT/US 96/04017

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0273085	06-07-88	EP-A-	0296222	28-12-88
		JP-T-	1501838	29-06-89
WO-A-9114696	03-10-91	AU-B-	7759291	21-10-91
		CA-A-	2079109	30-09-91
		EP-A-	0537299	21-04-93